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## Accepted Manuscript

The effect of food restriction on the regulation of gonadotropin-releasing hormone in male house finches (*Haemorrhous mexicanus*)

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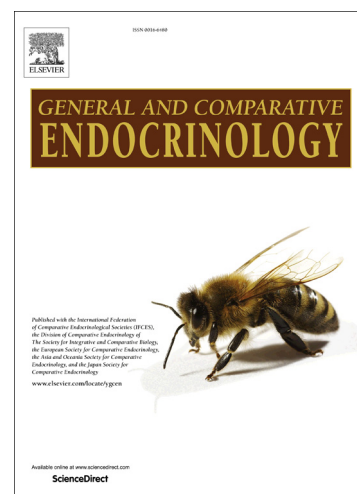
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21       **Highlights**  
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- 23       • Food-restricted birds have smaller testes but unaltered plasma testosterone.  
24       • Baseline plasma luteinizing hormone is marginally lowered by food restriction.  
25       • Food restriction enhances the secretory capacity of gonadotropin-releasing hormone.  
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54           **Abstract**

Seasonal activation of the vertebrate hypothalamic-pituitary-gonadal (HPG) axis and gonadal development is initiated by gonadotropin-releasing hormone-I (GnRH) release from the hypothalamus. In photoperiodic species, the consistent annual change in photoperiod is the primary environmental signal affecting GnRH cell activity, including changes in the synthesis and secretion of this neuropeptide. Non-photoperiodic environmental cues such as energy availability also influence HPG axis activity, but the mechanisms mediating this influence, in particular on the GnRH system, are unclear. Understanding how the neuroendocrine system integrates environmental information is critical in determining the plasticity and adaptability of physiological responses to changing environments. The primary objective of this study was to investigate GnRH-mediated changes in HPG axis activity and gonadal development in response to energy availability in a wild bird. We hypothesized that negative energy balance inhibits HPG axis activity by affecting GnRH secretion. Moderate food restriction for several weeks in male house finches, *Haemorrhous mexicanus*, decreased body condition and inhibited photoinduced testicular growth compared to birds fed *ad libitum*. Food restriction did not affect plasma luteinizing hormone (LH; a correlate of GnRH release) or plasma testosterone, but it enhanced the plasma LH response to an injection of the glutamatergic agonist, N-methyl-D-aspartate (NMDA). Thus, food restriction may decrease photoinduced HPG axis activation by acting centrally, in particular by attenuating the release of accumulated GnRH stores.

#### Keywords

Food restriction; luteinizing hormone; testosterone; gonadotropin-releasing hormone; passerine; seasonal breeding

#### Abbreviations

ANOVA: analysis of variance  
 AL: *ad libitum*  
 CP: cloacal protuberance  
 FR: food-restricted  
 FSH: follicle-stimulating hormone  
 GnIH: gonadotropin-inhibitory hormone  
 GnRH: gonadotropin-releasing hormone-I  
 GSI: gonadosomatic index  
 HPG: hypothalamic-pituitary-gonadal  
 ir: immunoreactivity  
 LH: luteinizing hormone

- 92 NMDA: N-methyl-D-aspartate  
93 Pro-GnRH: gonadotropin-releasing hormone precursor peptide  
94 T: testosterone  
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# 1. Introduction

Seasonal reproductive development in vertebrates is controlled through activation of the hypothalamic-pituitary-gonadal (HPG) axis. Environmental signals (in birds, primarily long days) stimulate gonadotropin-releasing hormone-I (GnRH) secretion from the hypothalamus (Dawson et al., 2001; Follett et al., 1977). GnRH stimulates the anterior pituitary gland to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH; Hattori et al., 1986; Sharp et al., 1987), which then act on the gonads to increase steroid hormone production and secretion; this action results in gonadal growth and gametogenesis (Deviche et al., 2011; Kirby and Froman, 2000). Steroid hormones, in turn, modulate HPG axis activity via negative feedback on the hypothalamus and pituitary gland (e.g. Deviche et al., 2006). The stimulating effects of GnRH are opposed by gonadotropin-inhibitory hormone (GnIH), which decreases GnRH and/or gonadotropin release in response to photoperiod and other environmental signals (e.g., Tsutsui et al., 2012).

The mechanisms by which photoperiod regulates the activity of the avian HPG axis have been extensively studied (Dawson, 2014; Yoshimura, 2013). In response to long days, many avian species that are strict seasonal breeders undergo a process of photostimulation during which the HPG axis is activated. Photostimulation is usually followed by photorefractoriness, during which continued long day exposure ultimately reduces HPG axis activity causing reproductive system regression (e.g., Hahn et al., 2009). In these species, photosensitivity and, therefore, the ability of long days to again stimulate the HPG axis, is reinstated after sufficient exposure to short days (e.g., Stevenson et al., 2012). Each level of the HPG axis is under some degree of independent regulation (Schaper et al., 2012; Stevenson et al., 2013; Williams, 2012), but photoperiod regulates HPG axis activity primarily by altering GnRH synthesis and secretion (Ball, 1993; Cho et al., 1998; Joseph et al., 2013; Nicholls et al., 1988). GnRH synthesis can be investigated by measuring the expression of its precursor peptide, proGnRH (Meddle et al., 2006a&b; Parry et al., 1997) or GnRH gene expression (Stevenson et al., 2013; Ubuka et al., 2009). GnRH secretion is not easily measured directly, but plasma LH can be used as a proxy of this secretion (Ball, 1993). N-methyl-D-aspartate (NMDA) is a neuroexcitatory amino acid glutamate analog which stimulates GnRH release (Meddle et al., 1999; Deviche et al., 2008; Iremonger et al., 2010), and the plasma LH increase that occurs in response to a NMDA injection can be used as indicator of the amount of releasable GnRH (Meddle et al., 1999; Stevenson et al., 2012). Photostimulation is associated with elevated GnRH synthesis and release, photorefractoriness is associated with a decline in GnRH release followed by a decline in synthesis, and photosensitivity with renewed synthesis (Bentley et al., 2013; Dawson and Goldsmith, 1997; Foster et al., 1987; Stevenson et al., 2009, 2012). Photosensitivity and photostimulation, therefore, differ with respect to GnRH transport and secretion.

Dependency of most middle and high latitude birds on photoperiod presumably evolved because of its reliability to predict seasonal increases in food supply and other optimal environmental conditions (Dawson and Sharp, 2007; Hahn et al., 2009). Reproductive success, and ultimately fitness, is generally

maximized by synchronizing breeding, and in particular, chick-rearing, with peaks in local food supply (Daan et al., 1990; Lack, 1968; Perrins, 1970). These peaks can vary inter-annually and in relation to the consistent annual photoperiodic cycle. Therefore, the ability to monitor and respond to factors associated with food availability and energy balance, by altering HPG axis activity, has the potential to enhance reproductive success (Visser et al., 1998). The use of food-related environmental cues in coordinating reproduction is evidenced in populations of free-living birds in which the timing of breeding varies inter-annually and between territories in relation to food supply (Caro et al., 2006; Korpimäki, 1987; Nager and van Noordwijk, 1995; Pereyra et al., 2005; Perrins and McCleery, 1989; Solonen, 2014). Experimental food supplementation in free-living birds also positively impacts clutch size and breeding success across species (Derbyshire et al., 2015; Roper et al., 2018; Ruffino et al., 2014). In domestic birds, food deprivation can affect all levels of the HPG axis including the hypothalamus (Ciccone et al., 2007; Kobayashi et al., 2002; Tanabe et al., 1981), however, investigations involving moderate food restriction similar to what birds experience naturally remain rare and have produced inconsistent results (Davies et al., 2015; Dawson, 1986; Hahn, 1995).

The hypothalamic GnRH system responds to non-photoperiodic environmental signals, but whether changes in GnRH release and/or synthesis are involved in this response is not entirely clear. Brain GnRH-immunoreactivity (*ir*) changes independently of photoperiod in equatorial rufous-collared sparrows, *Zonotrichia capensis* (Moore et al., 2006), and in response to social signals in European starlings, *Sturnus vulgaris* (Stevenson and Ball, 2009) and ring-necked doves, *Streptopelia capicola* (Mantei et al., 2008). The significance of these findings is, however, ambiguous because an increase in brain GnRH-*ir* may reflect either an increase in synthesis that outpaces the rate of secretion or decreased secretion and/or transport of the peptide. Thus, a measure additional to GnRH-*ir* is useful to clarify the mechanisms regulating GnRH release. In the opportunistically breeding rufous-winged sparrow, *Peucaea carpalis*, for example, monsoon-related factors influence GnRH-*ir* and proGnRH-*ir* concurrently (Small et al., 2007), indicating changes in both synthesis and release of GnRH. In a previous study, we found that moderate food restriction inhibits photo-induced gonadal development in male house finches, *Haemorrhous mexicanus*, and also increases GnRH-*ir* without affecting proGnRH-*ir* (Valle et al., 2015). These results suggest in this species that the inhibitory influence of food restriction on the HPG axis involves an inhibition of GnRH secretion (Foster et al., 1988; Lee et al., 1990). This mechanism may be adaptive: if HPG axis plasticity in response to local environmental conditions is important in the early stages of breeding, then altering GnRH secretion in response to these conditions without affecting GnRH synthesis may provide increased flexibility with respect to the onset of breeding. Elucidating how the neuroendocrine system integrates and responds to environmental information is crucial for understanding the capacity of organisms to cope with environmental changes through plasticity and/or adaptation of the HPG axis (Wingfield, 2015).

The primary objective of this study was to comprehensively investigate GnRH-mediated changes in HPG axis activity and gonadal development in response to food availability in captive wild birds. Based



on previous work (Valle et al., 2015), we hypothesized that food availability affects HPG axis activity by regulating GnRH secretion. To test this hypothesis, we investigated hypothalamic GnRH release and the capacity of the hypothalamus to release stored GnRH in food-restricted male house finches. We used plasma LH as a correlate of GnRH release, and the plasma LH response to a NMDA injection as an indicator of the hypothalamus capacity to release GnRH. If food availability affects GnRH release without affecting its production, we predicted that initial plasma LH would be lower in food-restricted birds than in *ad libitum*-fed birds, and a NMDA injection to these birds would increase plasma LH to the same extent as in *ad libitum*-fed birds. Results of our previous work also led us to hypothesize that food restriction does not alter the pituitary gland responsiveness to GnRH (Valle et al., 2015). We tested this hypothesis in the present study by measuring the plasma LH response to a GnRH injection. If the hypothesis is correct, we predicted that this treatment would increase plasma LH similarly in *ad libitum*-fed and in food-restricted finches.

## 2. Methods

All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee. All necessary permits to capture animals were obtained from the US Fish and Wildlife Service and the Arizona Game and Fish Department.

### 2.1. Capture and initial conditions

Adult male house finches (N=20) were caught in Tempe, AZ, USA (33.41° N, 111.91° W; elevation: 360 m a.s.l.) between 31 January and 8 February 2015, at which time they were naturally exposed to a non-photostimulatory (11L: 13D) light: dark cycle ([www.timeanddate.com](http://www.timeanddate.com)) and had regressed testes (Hamner, 1966). Birds were caught using food-baited traps, sexed based on plumage coloration, and aged based on plumage characteristics (Pyle, 1997). Only after-second year (i.e., hatched in 2013 or earlier) males were selected. Birds were transported to Arizona State University Animal Care Facilities, placed in visually isolated, individual cages at 25° C, and kept on a semi-natural photoperiod (11L:13D; lights on at 7:30 AM). Birds initially received sunflower seeds *ad libitum* but the diet was gradually changed over 10 days to Mazuri small bird breeding diet (PMI Nutrition International, Richmond, IN, USA) for the rest of the study.

### 2.2. Food Restriction and Photostimulation

The daily food consumption of each bird was measured over the course of 1 week. For this, each bird was given 10 g of Mazuri pellet diet each morning, and the amount remaining after 24 hours was measured. Food was placed in bowls that had only a small opening for the bird's head so that spillage

was minimized. We found previously that individual daily food intake is relatively constant and can be adequately estimated using the average intake over 7 days (Valle et al., 2015). On 28 February 2018 (day 1), birds were randomly divided into 2 groups (N = 10): (1) *ad libitum* food availability (AL; = controls) and (2) food-restricted (FR). Food-restricted birds received a daily ration of food equal to 70% of their individual *ad libitum* food intake (Valle et al., 2015) until the end of the study whereas control birds continued to receive food *ad libitum*. At this time (day 1), all birds were transferred to a moderately stimulatory day length (13L: 11D; lights on at 6:00 AM) for the remainder of the study (6 weeks). House finches regain photosensitivity by the end of October (Hamner, 1966) and were thus photosensitive at the time of the transfer.

### 2.3. Morphology

We weighed all birds daily ( $\pm 0.1$  g) beginning on the day prior to photostimulation and dietary manipulation (27 February 2018: day 0) and continuing for the remainder of the study. Body fat reserves, muscle stores, and cloacal protuberance width were determined on day 0 and at the middle (3 weeks) and end (6 weeks) of the study. The amount of furcular fat was visually estimated using a scale of 0–5 according to Helms and Drury (1960). As the pectoral muscles contain the largest store of proteins in birds, their size was estimated using a scale of 0–3, with 0 for concave pectoral muscles and a prominent keel and 3 for convex pectoral muscles that protrude above the keel (Salvante et al., 2007). Cloacal protuberance width ( $\pm 0.1$  mm) was measured using digital calipers.

### 2.4. Blood Sampling and Hormone Challenges

The effect of food restriction on the plasma LH response to a NMDA or a GnRH injection was investigated after 3–4 weeks of photostimulation. An initial blood sample (100  $\mu$ l; time 0: T0) was taken from the jugular vein of each finch into a heparinized microsyringe and immediately placed on ice. Each bird then received an intramuscular injection (i.m.) of either 1.2 mg NMDA (Sigma Chemical Co., MO, USA) or 1.25  $\mu$ g GnRH-I (Sigma Chemical Co., MO, USA) dissolved in 50  $\mu$ l sterile saline solution. After an injection, finches were returned to their cage and they were bled again (100  $\mu$ l) 20 minutes later (time 20: T20). The dose and sampling time for the NMDA injection are based on previous studies that found a stimulatory effect of NMDA on plasma LH. In white-crowned sparrows, *Zonotrichia leucophrys gambelii*, the LH response to subcutaneous NMDA injection was measured at 2, 8, and 20 minutes and the response was found to be highest at 20 minutes (Meddle et al., 1999). An i.m. NMDA injection, as used in this study, raised plasma LH in cassin's sparrows, *Peucaea cassinii*, after 15 minutes (Deviche et al., 2008). The dose and sampling time for the GnRH injection is based on our previous experiment in house finches, which found a stimulatory effect of i.m. GnRH injection on plasma T 30 minutes after injection (Valle et al., 2015). In zebra finches, *Taeniopygia guttata*, plasma LH levels were elevated 10 minutes

after intravenous GnRH injection in nonbreeding males (Perfito et al., 2011). We chose an intermediate sampling time (20 minutes) because GnRH was administered i.m. and samples were used to measure plasma LH and not T.

Each bird received both injections, 1 week apart, with the weekly sequence of injections divided equally between the treatment groups and the daily sequence randomized across all birds. All samples were collected between 9:00 and 11:00 AM. Samples were centrifuged within 3 hours of collection, and plasma was collected and stored at -80°C until assayed.

Additional blood samples for plasma LH and T determination were collected on day 0 and after 6 weeks of the treatment. At each time, blood (150 µl) was taken and plasma was stored as described above. Samples obtained during weeks 3 and 4 of treatment and before injection (T0) were also used to analyze unstimulated plasma LH throughout the study.

## 2.5. Euthanasia and Testis Measurement

After 6 weeks of photostimulation and dietary manipulation, and 2 weeks after the last injection, birds received an i.m. injection of 400 µl anesthetic solution (0.9% NaCl containing 20 mg/ml xylazine and 100 mg/ml ketamine). To preserve the brain for potential future immunohistochemical analysis, birds were perfused transcardially with 35 ml wash solution (0.9% NaCl and 0.1% NaNO<sub>2</sub> in 0.1 M phosphate buffer, PB) followed by 35 ml of fixative (4% paraformaldehyde and 0.1% NaNO<sub>2</sub> in 0.1 M PB). The testes were removed, rinsed in saline, and weighed to the nearest 0.1 mg. The individual gonadosomatic index (GSI) was calculated as testis mass as a percentage of the body mass.

## 2.6. Plasma LH and T Assays

### 2.6.1. Luteinizing Hormone (LH)

We used a validated radioimmunoassay (Sharp et al., 1987, with slight modifications) to measure plasma LH. This radioimmunoassay has been used to quantify plasma LH in many avian species (Ciccone et al., 2007; Davies et al., 2015; Deviche et al., 2012; Fraley et al., 2013; Meddle et al., 2002), including house finches (Salvante et al., 2013). Briefly, the assay reaction volume was 60 µl, comprised of 20 µl of plasma sample or standard, 20 µl of primary rabbit LH antibody and 20 µl of I<sup>125</sup>-labelled LH. The primary antibody was precipitated to separate free and bound I<sup>125</sup> label using 20 µl of donkey anti-rabbit precipitating serum and 20 µl of non-immune rabbit serum. All samples were assayed in duplicate in a single assay. The intra-assay coefficient of variation was 4.89% and the minimum detectable concentration was 0.15 ng/ml.

### 2.6.2. Testosterone (T)

A validated (Deviche and Cortez, 2005) commercial enzyme-linked immunoassay (Enzo Life Sciences, Farmingdale, NY, USA) was used to measure plasma T following the manufacturer's instructions. Plasma was diluted 15x in assay buffer containing 1 µl displacement reagent per 99 µl plasma. Samples were assayed in duplicate with all samples from each bird on a single assay plate. Each assay plate included a complete standard curve. The assay sensitivity was 4.81 pg/ml and the intra-assay coefficient of variation was 2.9% (N=39 samples).

## 2.7. Statistical Analyses

Effects of the dietary manipulation on body mass, morphological characteristics, and plasma hormones were analyzed using two-way repeated measures analysis of variance (ANOVA), with time (number of days) as the within-subject factor and food availability as the between-subjects factor. Effect of the dietary manipulation on testis mass and GSI was analyzed using Student's t-tests. Effects of GnRH or NMDA injection on plasma LH were analyzed using two-way repeated measures ANOVA with time (T0 vs. T20) as the within-subject factor and food availability as the between-subjects factor. For ordinal scale data (fat and muscle scores), data were ranked before proceeding with analyses. Data sets that were not normally distributed or homoscedastic (Shapiro-Wilk test and Levene's test, respectively) were either natural log- (plasma LH) or square root- (plasma T) transformed prior to analysis. The transformed datasets displayed normality and homoscedasticity. For data sets that did not display sphericity (body mass, baseline plasma LH), according to Mauchly's sphericity test, degrees of freedom were deflated using a  $\epsilon$ -derived Greenhouse-Geiser correction. When a statistically significant treatment x time interaction was detected using ANOVA, pair-wise comparisons were performed using Bonferroni post hoc tests. Data were analyzed using SPSS (version 24; IBM, Armonk, NY, USA). Graphs were made using Graphpad Prism 8 (La Jolla, CA, USA) and present untransformed data.

Three birds (1 FR and 2 AL) died during the experiment, resulting in the absence of data for 2 birds after 3 weeks and 3 birds after 4 weeks. Additionally, we were unable to collect a T20 blood sample after NMDA injection from one bird, and to obtain enough blood to measure baseline LH from one bird at day 0 and another at the end of the study. We estimated missing values using multiple imputation (MI) and the NORM program (<http://sites.stat.psu.edu/~jls/misoftwa.html>; Schafer, 1999). Multiple imputation relies on more plausible assumptions than other approaches to coping with missing data (e.g., case deletion or replacement with group means), properly accounts for uncertainty about missing values (leading to appropriate standard errors), and retains original sample sizes (Little and Rubin, 2002).

## 3. Results

### 3.1. Body Condition

Body mass was affected by food availability ( $F_{1,15} = 11.37$ ,  $P = 0.004$ ) and time ( $F_{3,47} = 14.08$ ,  $P < 0.001$ ), and there was an interaction between these factors ( $F_{3,47} = 23.67$ ,  $P < 0.001$ ; Fig. 1A). *Ad libitum*-fed and food-restricted birds had similar body mass at the start of the dietary manipulation ( $P = 0.82$ ) and AL birds experienced minor fluctuations in body mass. FR birds lost mass within the first week of food restriction and maintained lower body mass than AL birds for the duration of the study ( $P < 0.002$ ).

Furcular fat scores were affected by food availability ( $F_{1,18} = 15.60$ ,  $P = 0.001$ ) and there was a food availability x time interaction ( $F_{2,36} = 15.49$ ,  $P < 0.001$ ; Fig. B), with no effect of time alone ( $F_{2,36} = 2.00$ ,  $P = 0.15$ ). *Ad libitum*-fed birds had more furcular fat 6 weeks into the experiment than at the start ( $P < 0.001$ ), whereas FR birds lost fat stores after 6 weeks of food restriction and had less fat than AL birds 3 and 6 weeks after treatment onset ( $P < 0.008$ ).

Pectoral muscle size was affected by food availability ( $F_{1,18} = 18.49$ ,  $P < 0.001$ ) and there was a food availability x time interaction ( $F_{2,36} = 22.90$ ,  $P < 0.001$ ; Fig. 1C), with no effect of time alone ( $F_{2,36} = 2.65$ ,  $P = 0.08$ ). Pectoral muscle size increased in AL birds after 6 weeks of dietary manipulation ( $P < 0.008$ ) but decreased after 3 weeks of treatment in FR birds ( $P = 0.01$ ), with smaller pectoral muscles in these birds compared with AL birds at 3 and 6 weeks ( $P < 0.001$ ).

### 3.2. Cloacal Protuberance

Cloacal protuberance (CP) width differed between food treatment groups over the course of the study ( $F_{2,36} = 5.55$ ,  $P = 0.008$ ; Fig. 1D). It increased in AL birds after 3 weeks of exposure to long days, remaining at this size after 6 weeks ( $P < 0.005$ ). In FR birds, CP width was not affected by long day exposure ( $P > 0.38$ ), and was lower in FR than AL birds after 3 weeks of dietary manipulation ( $P = 0.04$ ). There was a main effect of time ( $F_{2,36} = 12.69$ ,  $P < 0.001$ ) and no effect of food availability alone ( $F_{1,18} = 1.47$ ,  $P = 0.076$ ).

### 3.3. Testis Mass and Plasma T

Food-restricted birds had lower paired testis mass ( $T_{15} = -4.7$ ,  $P < 0.001$ ) and GSI ( $T_{13,4} = 4.58$ ,  $P < 0.001$ ; Fig. 2) than AL birds.

Baseline plasma T decreased during the period of dietary manipulation ( $F_{1,18} = 8.50$ ,  $P = 0.009$ ), but was unaffected by food availability ( $F_{1,18} = 0.96$ ,  $P = 0.34$ ), and there was no food availability x time interaction ( $F_{1,18} = 0.43$ ,  $P = 0.52$ ; Fig. 3B).

### 3.4. Plasma LH

Baseline plasma LH changed over time in both AL and FR birds ( $F_{2,34} = 9.39$ ,  $P = 0.001$ ), increasing above initial levels after 3 and 4 weeks before declining after 6 weeks ( $P < 0.02$ ). There was a

marginal effect of treatment, with baseline plasma LH lower overall in FR as compared to AL birds, but this difference did not reach significance ( $F_{1,18} = 4.02$ ,  $P = 0.06$ ). There was no interaction between time and food availability on baseline plasma LH ( $F_{2,34} = 0.73$ ,  $P = 0.48$ ; Fig. 3A).

Plasma LH increased in response to GnRH challenge ( $F_{1,18} = 199.50$ ,  $P < 0.001$ ), but this increase was unaffected by food availability ( $F_{1,18} = 0.18$ ,  $P = 0.68$ ), and there was no interaction between the effect of GnRH challenge and food availability on plasma LH ( $F_{1,18} = 1.55$ ,  $P = 0.23$ ; Fig. 4A). This conclusion is supported by examination of the fold increase in LH following GnRH challenge, as this increase did not differ in AL and FR finches ( $T_{18} = -1.24$ ,  $P = 0.23$ ; Fig. 4C).

Plasma LH increased in response to NMDA challenge ( $F_{1,18} = 131.63$ ,  $P < 0.001$ ). There was a significant interaction between the effects of food availability and NMDA challenge on plasma LH ( $F_{1,18} = 4.69$ ,  $P = 0.044$ ; Fig. 4B). Plasma LH in FR birds did not differ significantly from plasma LH in AL birds prior to ( $P = 0.093$ ) or after NMDA-challenge ( $P = 0.97$ ). However, the fold increase in plasma LH after NMDA injection was approximately twice as large in FR (6X) compared to AL birds (3X;  $T_{18} = 2.17$ ,  $P = 0.04$ ; Fig. 4D).

#### 4. Discussion

We tested the hypothesis that food availability affects photoinduced HPG axis activity and gonadal growth by regulating GnRH secretion. Baseline GnRH secretion was estimated by measuring plasma LH in intact birds and we predicted that initial plasma LH would be lower in FR than in AL birds. We determined the potential to secrete GnRH by measuring the plasma LH response to a NMDA challenge and predicted that in response to this challenge, plasma LH would increase to a similar level in AL and FR birds, i.e., that in relative terms it would increase more in FR than in AL finches. Food restriction decreased body condition and resulted in smaller testes and diminished CP growth, but had no effect on baseline plasma T or LH. However, plasma LH increased more in FR than AL birds in response to a NMDA challenge. These results are consistent with the hypothesis that food restriction did not alter basal GnRH secretion but enhanced the capacity to secrete GnRH, thereby suggesting that a main effect of this manipulation is to inhibit GnRH release from the hypothalamus.

##### 4.1. Testis development and function under food restriction

The inhibition of photoinduced testicular development under food restriction is consistent with our previous findings (Valle et al., 2015). Smaller testes in FR than AL birds likely were associated with lower levels of spermatogenesis, as we found smaller seminiferous tubules in FR house finches previously (Valle et al., 2015). Seasonal testicular growth is stimulated primarily by FSH, but additionally by LH and T (Deviche et al., 2011). We found no effect, however, of 6 weeks of food restriction on plasma T. Testosterone was lowered by 3-4 weeks of food restriction in our previous study, but only transiently



during photostimulation, with differences disappearing after 6 weeks (Valle et al., 2015). Several studies on other avian species found a negative effect of food restriction on plasma T (Lynn et al., 2010; Lynn et al., 2015; Perez-Rodriguez et al., 2006) and body condition is positively related to plasma T in free-living house finches (Duckworth et al., 2001). It is, therefore, possible in the present study that food restriction decreased plasma T, but not at the times that blood samples were collected to measure plasma levels of the steroid. Supporting the hypothesis that plasma T was transiently lowered in FR birds, we found that CP growth, a T-dependent trait (Deviche and Cortez, 2005), occurred in AL birds in response to photostimulation, but did not occur in FR birds.

#### 4.2. Baseline plasma LH under food restriction

Baseline plasma LH increased in response to photostimulation, but this increase was unaffected by food restriction. If food availability controls gonadal development by affecting GnRH and subsequently gonadotropin release and gonadal stimulation, we predicted that smaller testes in FR than AL birds would be associated with a parallel difference in plasma LH. Food-restricted birds did have smaller testes, but this did not co-occur with lower plasma LH. In the few studies that have measured both plasma LH and gonadal growth in wild birds under food restriction, that parallel changes actually do not appear common. For example, the food restriction-induced decline in plasma LH in Abert's towhees was not associated with a decrease in gonadal growth (Davies et al., 2015) and in the red crossbill, *Loxia curvirostra*, held on long days, testis growth, but not LH, was inhibited by food restriction (Hahn, 1995). Baseline plasma LH in the present study was marginally lower overall in FR than AL finches, and it cannot be discounted that a small reduction in plasma LH (and potentially FSH) under food restriction suffices to inhibit testicular development significantly.

#### 4.3. LH responsiveness under GnRH and NMDA challenge

Our previous study found no differential responsiveness of the pituitary gland to GnRH under food restriction, as measured by plasma T, and also no effect of this treatment on LH-induced plasma T (Valle et al., 2015). The present findings, showing that GnRH stimulated LH release and this stimulation was not treatment-related, confirm that food restriction does not attenuate the LH responsiveness to GnRH. Consistent with previous studies, baseline plasma LH in the present study, therefore, can serve as an indicator of GnRH release, and the LH response to NMDA reveals the potential of hypothalamic GnRH neurons to secrete this neuropeptide. In this context, we can conclude that food restriction resulted in a marginal reduction in basal GnRH release. It should be noted that the plasma LH response to GnRH injection may not have been measured when this response was greatest. Furthermore, birds were sampled once after GnRH injection and so the experimental design could not inform on potential differences between groups in the time course of GnRH effects on plasma LH.

Administration of NMDA increased plasma LH across all birds. Food restriction resulted in an enhanced response to NMDA injection, with a greater increase in plasma LH from initial levels in FR than AL finches. The LH response to NMDA in seasonally breeding birds varies as a function of the photoperiodic state, with the largest response occurring during photosensitivity, a moderate response under photostimulation, and barely any response during photorefractoriness (Dawson, 2005; Deviche et al., 2008). In photosensitive birds, photostimulation stimulates the release of GnRH synthesized under short days (Stevenson et al., 2012). It is possible that the photostimulatory conditions in the present study were not sufficient to override the inhibitory effect of food restriction on basal GnRH release, in which case FR birds would have had larger stores of GnRH that they could release in response to NMDA stimulation than AL birds. Greater GnRH-*ir* indicates larger stores of GnRH (Foster et al., 1988). We did not measure these stores in the present study, but found previously that GnRH-*ir* was higher in FR house finches exposed to nearly identical conditions as in the present study than in AL finches (Valle et al., 2015). Taken together, these studies support the conclusion that a negative energetic state, as induced here by food restriction, results in elevated store of releasable GnRH.

The LH response to both peripheral and central injections of NMDA has been used extensively in birds and other vertebrates as an indirect measure of GnRH responsiveness (Cicero et al., 1988; de Tassigny et al., 2010; Dawson et al., 2005; Deviche et al., 2008; Meddle et al., 1999; Iremonger et al., 2010). Glutamatergic activation is not specific to GnRH neurons and the peripheral NMDA administration used here may have stimulated LH release through mechanisms not involving GnRH. Available evidence, however, indicates that GnRH neurons mediate the secretion of LH that follows NMDA administration. In particular, the LH response to peripherally administered NMDA in rats is prevented by treatment with a GnRH receptor antagonist (Cicero et al., 1988). Furthermore, in mice a similar increase in LH secretion occurs in response to either central or peripheral administration of NMDA, with both administration routes apparently affecting GnRH release, albeit through different pathways (de Tassigny et al., 2010). Peripheral administration of NMDA, as used in the present study, has been demonstrated in both mammals and birds, to primarily affect GnRH release through enhancing activity in the region of GnRH nerve terminals, as determined by the quantification of c-Fos-*ir*, (de Tassigny et al., 2010; Deviche et al., 2008; Meddle et al., 1999).

Multiple types of evidence, primarily from mammals, demonstrate how food availability might modify GnRH release. Food availability appears to primarily affect GnRH activity in the median eminence, the region of its release (Temple and Rissman, 2000). It affects both thyroid hormones (Costa-e-Sousa, 2012; Darras et al., 1995; Herwig et al., 2009) and hypothalamic deiodinase expression (Herwig et al., 2009), both of which influence photoinduced morphological changes in glial cells that surround GnRH terminals in the median eminence and regulate its release (Yamamura et al., 2004; Yoshimura et al., 2003). Recent evidence actually links regulation of gonadal growth by food availability to altered glial cell activity in proximity to GnRH nerve terminals (Steinman et al., 2012). Gonadotropin-inhibitory hormone (GnIH) activity may also play a role in regulating GnRH release under food restriction. Indeed, GnIH



activity under some circumstances relates to feeding (Clarke et al., 2012; Davies et al., 2015; Fraley et al. 2013) and in European starlings, GnIH can modulate the effect of other non-photoc factors to GnRH cells (Calisi et al., 2011). We did, however, find no change in GnIH-ir in FR house finches (Valle et al., 2015). As NMDA acts primarily to stimulate GnRH nerve terminals (Deviche et al., 2008; Meddle and Follett, 1997; Meddle et al., 1999), its stimulatory effect on plasma LH, and presumably GnRH release, may consist in overriding these mechanisms inhibiting GnRH release under food restriction.

#### 4.4. Conclusion

This study is among the first to investigate the regulation of GnRH release by food availability in a wild, although captive, bird. Food restriction inhibited photoinduced gonadal development and this inhibition may involve attenuated photoinduced GnRH release. We propose that a lower energetic state under food restriction decreases basal GnRH release, thereby elevating neuronal GnRH stores and resulting in enhanced GnRH and, therefore, LH secretion during pharmacological stimulation. In birds that naturally experience fluctuating and unpredictable environmental conditions, such plasticity in HPG axis activity is crucial for making decisions about allocating energy towards reproduction or survival. Continued investigation into the central and peripheral mechanisms by which animals integrate energetic information will help to better understand the plasticity of breeding responses and ultimately how populations succeed or fail in adjusting to environmental changes.

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#### 5.5 Declarations of Interest

None

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# Figure Legends

**Figure 1.** Effects of food restriction on body condition and cloacal protuberance width in male house finches, *Haemorrhous mexicanus*. Body mass (**A**) was reduced by food restriction after 1 week and remained lower than *ad libitum*-fed birds. Both furcular fat score (**B**) and pectoral muscle score (**C**) were reduced by 6 weeks of food restriction to a smaller size than control birds. Cloacal protuberance width (**D**) increased in response to long day exposure (beginning at day 0), but did not increase in size in food-restricted birds, resulting in a smaller CP after 3 weeks in food-restricted birds than in *ad libitum*-fed birds. Data are plotted as means  $\pm$  SEM. An asterisk (\*) indicates a significant difference between treatment groups ( $P < 0.05$ ; Bonferroni post-hoc tests). For visual clarity, some points have been separated along the horizontal axis.

**Figure 2.** Food restriction for 6 weeks reduces paired testis mass (**A**) in photostimulated male house finches. Gonadosomatic index (testis mass as a percentage of body mass) was also lower (**B**) in food-restricted birds as compared to birds fed *ad libitum*. Data is plotted as means  $\pm$  SEM, and the asterisk denotes a significant difference between the groups ( $P < 0.05$ ; Student's t-test).

**Figure 3.** Baseline plasma luteinizing hormone (LH) and testosterone (T) are unaffected by food restriction in male house finches. Plasma LH (**A**) changed over the duration of the study, initially increasing in response to photostimulation. This response was similar between food-restricted and *ad libitum*-fed birds, being marginally lower in food-restricted birds compared to control birds ( $P = 0.06$ ; ANOVA). Plasma T (**B**) was lower after 6 weeks, but similar in both food-restricted and *ad libitum*-fed birds. Data are plotted as means  $\pm$  SEM.

**Figure 4.** Food restriction differentially affects the increase in plasma luteinizing hormone (LH) that occurs in response to a gonadotropin-releasing hormone (GnRH) or N-methyl-D-aspartate (NMDA) challenge in male house finches. GnRH challenge (**A**) increased plasma LH similarly in food-restricted birds as compared to *ad libitum*-fed birds, with the percent change in LH (**C**) being similar between groups. Food restriction enhanced (**B**) the increase in plasma LH that occurred in response to a NMDA challenge ( $P = 0.04$ ; ANOVA) with food-restricted birds having a greater percent change (**D**) than *ad libitum*-fed birds. Data are shown as means  $\pm$  SEM, and the asterisk denotes a significant difference between the groups ( $P < 0.05$ ; Student's t-test).

Figure 1.

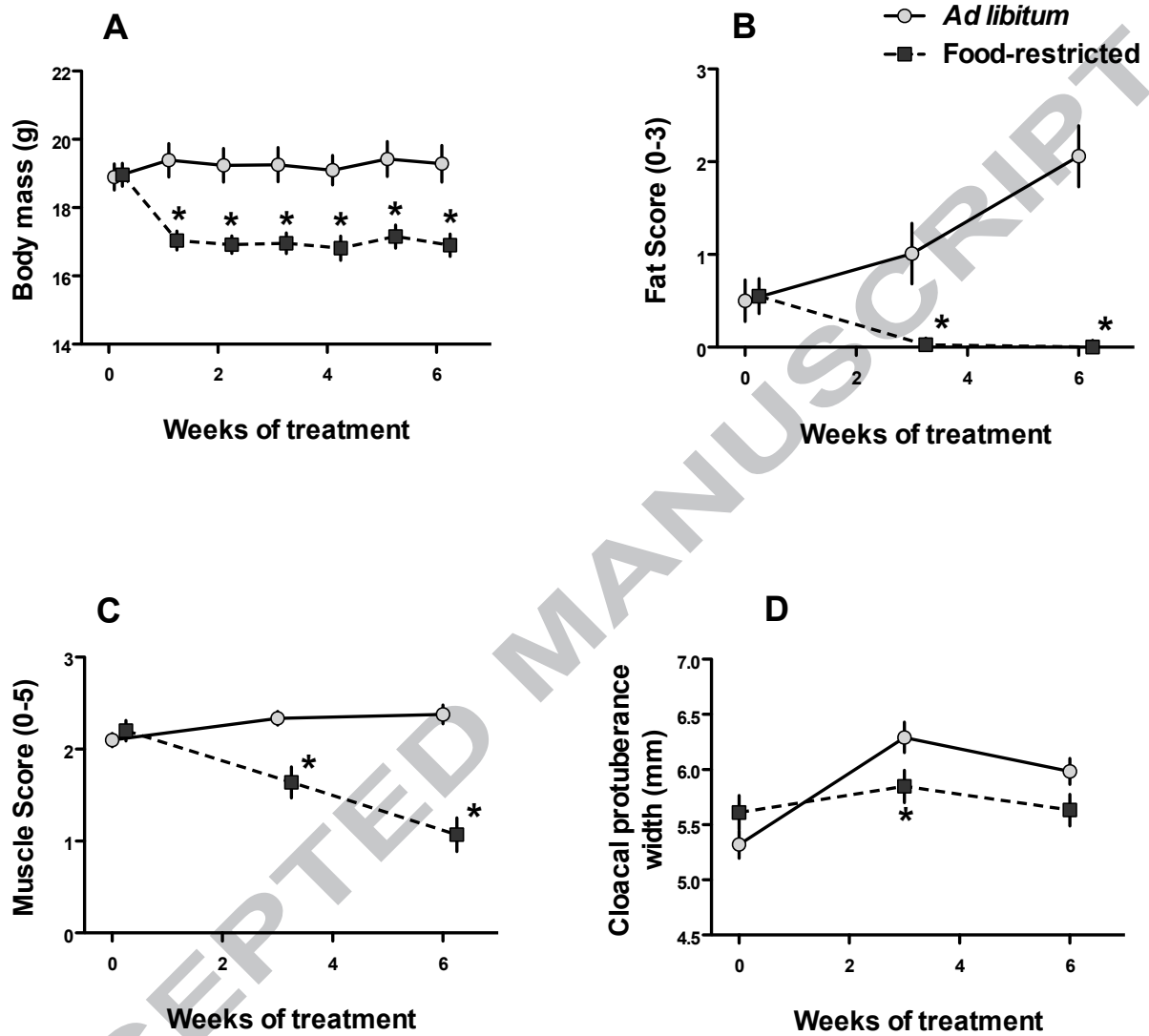


Figure 2.

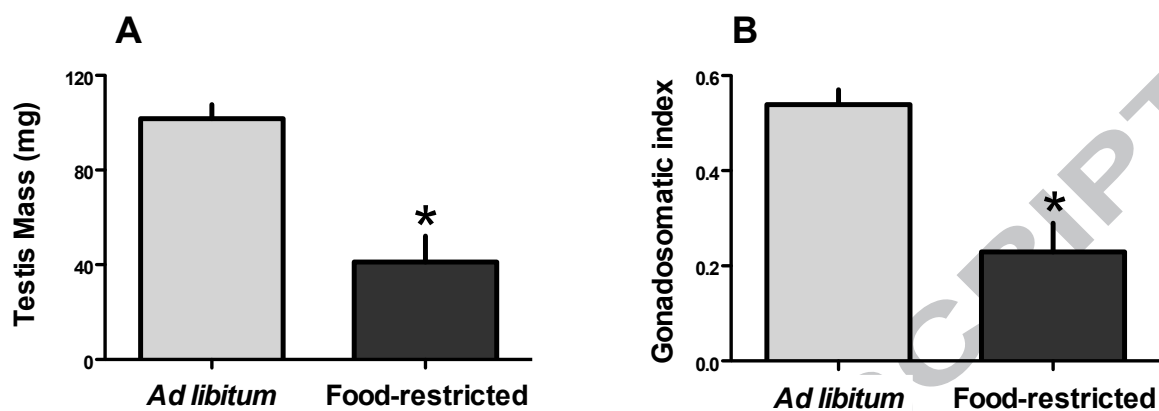
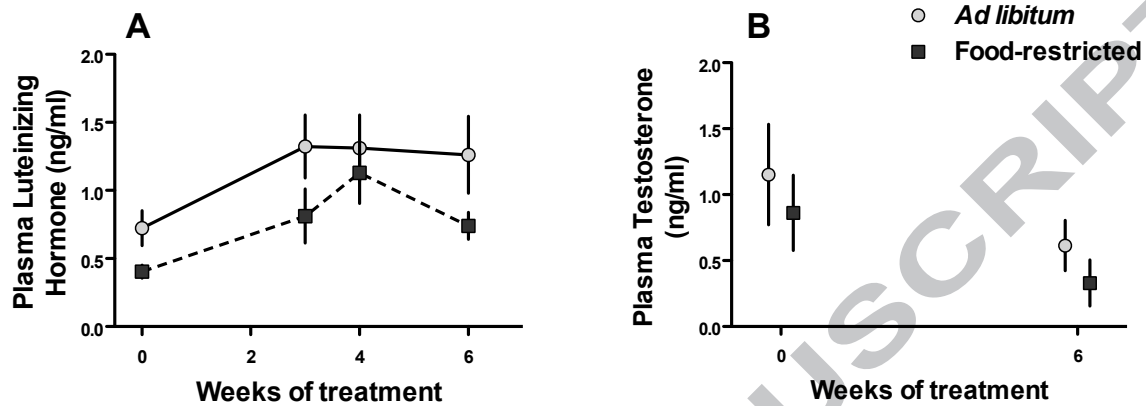
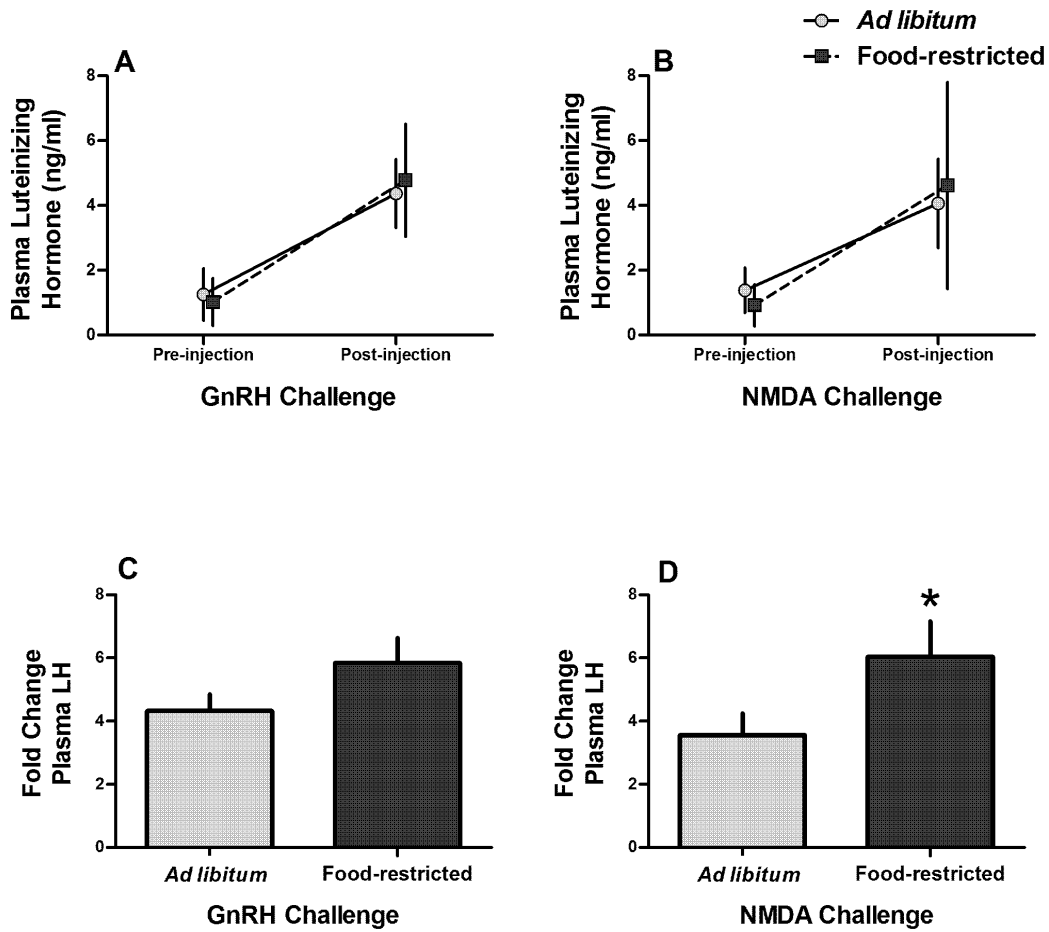


Figure 3.



784 **Figure 4.**



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